Conditional disruption of synaptic transmission induces male–male courtship behavior in *Drosophila*

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It is reported here that male–male courtship behavior is evoked instantaneously in the fruit fly *Drosophila* by conditional disruption of synaptic transmission. A temperature-sensitive allele of the *Drosophila* dynamin gene *shibire* (*shi^{ts1}*) was expressed by using the GAL4/UAS system to disrupt synaptic transmission from GAL4-positive neurons in a temperature-dependent manner. An enhancer-trap GAL4 line C309 directing *shi^{ts1}* expression in central and peripheral neurons (C309/UAS-*shi^{ts1}*) initiated stereotypical precopulatory behavior toward other mature males immediately after a temperature shift from the permissive to restrictive temperature. At the restrictive temperature, C309/UAS-*shi^{ts1}* males formed ''courtship chains'' and exhibited abnormally high levels of head-to-head interactions. The temperature-induced male–male courtship is attributable not to an increase in sexual attractiveness but to an increase in sexual activity of C309/UAS-*shi^{ts1}* males. Interestingly, the temperature-induced increase in sexual activity is specific toward male partners, because C309/UAS-*shi^{ts1}* males courted receptive virgin females less vigorously and copulated less efficiently after shifted to the restrictive temperature. Among the GAL4-positive neurons in C309, conditional disruption of certain cholinergic neurons but not the mushroom body intrinsic neurons plays a critical role in the induction of male–male courtship. These neurons may be involved in inhibitory systems that normally suppress aberrant male–male courtship. The presented strategy that can induce behavioral abnormalities by disrupting synaptic transmission in an acute and noninvasive manner will allow further exploration as to how distinct neuronal groups control sexual orientation and other aspects of reproductive behavior in *Drosophila*.

Sexual orientation of the fruit fly *Drosophila* has a genetic basis, which is evidenced by particular genetic variants that exhibit aberrant bisexual orientation (1, 2). Viable mutant alleles of the *fruitless* gene (*fru*) show a variety of courtship abnormalities including vigorous male–male courtship (3–5). When male *fru* mutants are grouped together, they form ''courtship chains'' in which a courting male is courted by other males, leading to a line of flies (3). *fru* encodes sex-specific proteins belonging to the BTB/zinc-finger family of transcription regulators that specify aspects of sexual differentiation in the central nervous system (CNS) under the regulation of the *transformer* (*tra*) gene (6, 7). Other genetic variants having defects in recognizing appropriate mates include mutants for *dissatisfaction* (*dsf*) (8, 9) and *quick-to-court* (*qtc*) (10) as well as the *Voila^{1}* genetic variant that carries a P[GAL4] transposon insertion within the promoter of the *prospero* gene (11, 12). Vigorous male–male courtship is observed also when the wild-type product of the *white* gene (w^{+}) is expressed ubiquitously under the control of the heat-shock promoter (13, 14). Although the nature of these genetic variants is characterized at a molecular level, the mechanistic basis as to how these genes are involved in determining sexual orientation remains elusive. This is partly because little is known about the neuronal circuitry that controls the actual manifestation of male reproductive behavior.

In an attempt to define brain regions involved in male reproductive behavior, male flies with regionally feminized brains were generated by using the classical XX/XO mosaic technique (15, 16) or expressing the female form of *tra* (*tra^{F}*) in a limited number of neurons under the control of GAL4 enhancer-trap lines (17, 18). Flies courted males as well as females when *tra^{F}* was expressed in part of either the antennal lobes (ALs), which receives olfactory information, or the mushroom bodies (MBs), higher brain regions that process olfactory information from the AL. It has been suggested that feminization of the olfactory system may destroy a male fly's ability to detect and/or process information obtained from the volatile chemical compounds that play an important role in mate discrimination. These male/female mosaic approaches have shown clearly that certain neurons localized to defined substructures of the brain are critical for reproductive behavior. However, the use of sex mosaics to define centers and circuits responsible for courtship behavior is somewhat limited, because the neurons playing a key role in courtship are not necessarily sexually dimorphic. In addition, there is a possibility that *tra*-induced feminization of particular neurons may alter anatomical features and/or physiological properties of neurons interacting with *tra*-expressing neurons in the course of development.

I recently developed a method for conditionally disrupting synaptic transmission of anatomically defined neurons in the intact nervous system by directing expression of a temperature-sensitive allele of the *Drosophila* dynamin gene *shibire* (*shi^{ts1}*) by using the GAL4/UAS system (19). The method has been used to induce paralysis, blindness, and memory defects in *Drosophila* in a temperature-dependent and GAL4 line-specific manner (19–22). In this paper, it is demonstrated that vigorous male–male courtship is evoked by *shi^{ts1}*-mediated conditional disruption of synaptic transmission, which is a useful way to explore the neuronal substrates underlying sexual orientation and other aspects of reproductive behavior in *Drosophila*.

Materials and Methods

***Drosophila*.** Flies were reared at 19°C under the conditions described in ref. 19 except where indicated otherwise. For UAS-*shi^{ts1}* (19) a third chromosome-linked line (line 10) was used exclusively in this study. The GAL4 lines C309 and C747 were obtained from Joshua Dubnau (Cold Spring Harbor Laboratory), and 17d and c492b were from Troy Zars (Theodor Boveri Institut fur Biowissenschaften, Würzburg, Germany). The OK107 GAL4 line and UAS-GFP line were obtained from the Bloomington Stock Center (Bloomington, IN). For the *Cha$^{3.3kb}$*-GAL80 construct, the *lacZ* portion of pCaSpeR-3.3kb-*lacZ* (23) was replaced with the GAL80 cDNA (obtained from Liqun Luo, Stanford University, Stanford, CA) as a *Bam*HI–*Xba*I fragment. *Cha$^{3.3kb}$*-GAL80 transformant lines were established by the procedure described in ref. 23.

Abbreviations: AL, antennal lobe; MB, mushroom body.

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Microscopy. The adult male brains and body parts were fixed with PBS containing 3.7% formaldehyde for 15 min at room temperature. GFP fluorescence was observed with a confocal microscope (Zeiss LSM 410). Illumination was at 488 nm (argon laser), and emission was at 515 nm. Z sections were collected at 2-μm intervals and processed to construct projections through an extended depth of focus. Images were processed minimally by using PHOTOSHOP (Adobe Systems, Mountain View, CA) to correct light levels, contrast, and brightness.

Behavioral Analysis. Courtship chaining behavior and head-to-head interactions were quantified by the methods described in refs. 5 and 24 with minor modifications. Male flies were collected within 8 h of eclosion under CO_2 anesthesia and stored in groups of five at 19°C. Five 4-day-old male flies were aspirated into Petri dishes (35 × 10 mm) prewarmed or cooled to observation temperature (30 or 19°C). The dishes were humidified with a circular piece of moistened Whatman 3MM paper. Flies were undisturbed for 20 min under a fluorescent lamp (13 W, maintained at a distance of 25 cm from the flies), then continuously videotaped for 10 min by using a Sony Digital-8 camera. Chaining index was measured as the percentage of a 10-min observation period during which courtship chains were observed (5). In this experiment, a courtship chain was defined as a group of more than three flies interacting with each other where at least two of them exhibited courtship behavior. A head-to-head interaction was not considered as courtship behavior for this analysis. From the same recordings, head-to-head interaction index was determined as the percentage of a 10-min observation period during which at least one pair of flies showed head-to-head interactions.

For observation of courtship behavior in pairs of flies, courtship chambers (8 × 3 mm) were used. Test males were collected as described above and stored individually in small food tubes (12 × 75 mm) at 19°C. Their mating partners [*white* (*w*) mutant males and females] were collected and stored in groups of 10 in vials at 19°C. A 5-day-old test male and a 4- or 5-day-old mate were aspirated into a courtship chamber and left undisturbed under a fluorescent lamp at 30 or 19°C for 3 min. The flies were videotaped for the following 5 min, and the courtship-index value (5, 25) was determined as the percentage of time that the subject male spent courting (i.e., following, tapping, singing, licking, attempted copulation, and copulation) during a 5-min observation period. In this experiment, simple orientation toward the partner was not included, because it was sometimes difficult, under the conditions used, to judge whether the males voluntarily oriented their bodies toward the partner. To determine mating success rates of appropriate male/female pairs, males and females were collected within 8 h of eclosion. The former were stored individually in small food tubes, and the latter were stored in groups of 10 at 19°C. Male/female pairs were aspirated into small food tubes and observed for 1 h at 30 or 19°C. A mating success rate was defined as the percentage of single male and female pairs that copulated in the 1-h observation period.

Results

An Enhancer-Trap GAL4 Line C309 Directs Gene Expression in the CNS and Peripheral Nervous System. When GFP reporter gene expression was driven by an enhancer-trap GAL4 line C309 (20, 21, 26, 27), strong GFP fluorescence was observed in all lobes (α, β, and γ), peduncles, and calyces of the MB as well as the cell body layer of the MB intrinsic neurons, Kenyon cells (Fig. 1 *A* and *B*). Besides the MB, GFP expression was seen in the pars intecerebralis, the AL, the optic lobes, the central complex, and the suboesophageal ganglion (ref. 26; Fig. 1 *A* and *B*). Some neuronal subsets in the thoracic ganglia of line C309 were labeled as described in ref. 26.

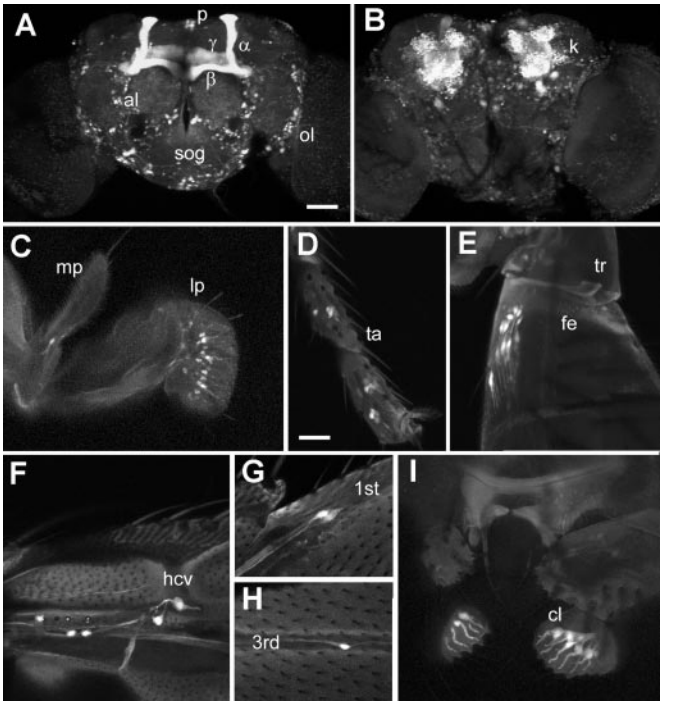

Fig. 1. GAL4 expression patterns of line C309 visualized by a GFP reporter gene. Distinct expression is observed in widespread regions of the adult brain (frontal view, *A*; posterior view, *B*) including α, β, and γ lobes of the MBs, the cell body layer of Kenyon cells (k), the pars intecerebralis (p), the AL (al), the optic lobes (ol), and the suboesophageal ganglion (sog). GFP expression is seen also in restricted sensory neurons in the labial palps (lp) (*C*), the tarsus (ta) (*D*), and the femur (fe)–trochanter (tr) junctions (*E*) of legs, the proximal radius of wings near the humeral cross-vein (hcv) (*F*), the first and third longitudinal wing veins (*G* and *H*), and the clasper (cl) of the male external genitalia (*I*) but not in the maxillary palp (mp) (*C*). [Scale bars: *A*, 50 μm (applied to *B* and *C*); *D*, 25 μm (applied to *E–I*).]

In addition to the CNS, line C309 also directed GFP reporter gene expression in limited groups of sensory neurons associated with putative contact chemoreceptors and mechanoreceptors. In each labial palp of the proboscis, a subset of gustatory neurons that were associated with different taste bristles (28) showed distinct GFP expression (Fig. 1*C*). In contrast, no detectable reporter gene expression was observed in olfactory neurons of the third antennal segment and the maxillary palp (Fig. 1*C*). In tarsal segments of the legs, a subset of cells associated with the taste bristles (28) were GFP-positive (Fig. 1*D*). Expression was observed also in neurons associated with chordotonal organs at the femur–trochanter junctions (ref. 29; Fig. 1*E*) but not in those innervating the tactile bristles distributed over the entire leg surface (30). In the wings, GFP expression was present in neurons associated with sensilla at the base of the radius (Fig. 1*F*) as well as in the first and third longitudinal veins (ref. 31; Fig. 1 *G* and *H*). In the male external genital organ, GFP expression was detected at the base of bristles in the claspers, which are thought to be mechanoreceptors (ref. 32; Fig. 1*I*).

C309/UAS-*shi^{ts1}* Males Show Temperature-Induced Male–Male Courtship Behavior. It was noticed that the mature male progeny of an enhancer-trap line C309 and UAS-*shi^{ts1}* (C309/UAS-*shi^{ts1}*) courted each other after they were transferred from 19 to 30°C. The temperature-induced male–male courtship activities included orientation toward a male partner, following him, tapping the partner's abdomen with the forelegs (Fig. 2*A*, arrow), unilateral wing vibration (Fig. 2*B*, arrow), and licking of the partner's genitalia (Fig. 2*C*, arrow). Attempted copulation (curl-

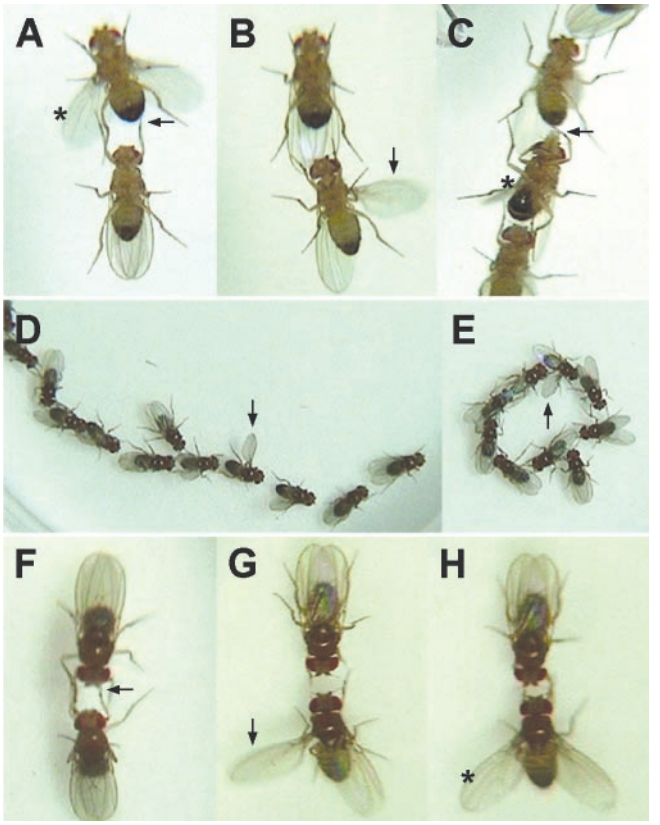

Fig. 2. High levels of intermale interactions in populations of C309/UAS-*shi*^{ts1} males evoked by a temperature shift from 19 to 30°C. C309/UAS-*shi*^{ts1} males display a stereotyped sequence of actions toward other males including tapping (*A*, arrow), unilateral wing vibration (*B*, arrow), and licking and abdominal curling (*C*, arrow and asterisk, respectively). Note that the courted male in *A* is flicking both of his wings as a possible rejection response (*A*, asterisk). A group of C309/UAS-*shi*^{ts1} males courting one another form a courtship chain (*D*) and ring (*E*). Arrows indicate flies exhibiting unilateral wing vibration. Shown are head-to-head interactions observed in populations of C309/UAS-*shi*^{ts1} males, in which a male taps at another male's head (*F*, arrow) and extends one (*G*, arrow) or both (*H*, asterisk) of his wings.

ing the abdomen to achieve genital–genital contact) was observed also (Fig. 2*C*, asterisk), but its occurrence was less frequent than other components of precopulatory behavior. Males courted by other males showed apparent rejection by flicking both of their wings together (Fig. 2*A*, asterisk) or kicking the courting male's head. However, C309/UAS-*shi*^{ts1} males that were being courted also showed vigorous courtship activity toward other males, leading to characteristic courtship "chains" (Fig. 2*D*) and "rings" (Fig. 2*E*), which have been observed in populations of *fru* mutants and flies ectopically expressing w^+ (1, 13).

The onset of the male–male courtship after a temperature shift was rapid. When five C309/UAS-*shi*^{ts1} males were transferred into a Petri dish (35 × 10 mm) prewarmed to 30°C, the unilateral wing vibration toward other males and the chain formation (see *Materials and Methods*) were first observed in a few minutes (143 ± 20 and 272 ± 25 sec, respectively; average ± SEM, $n = 25$). The effect of a temperature shift on male courtship was reversible. When the flies were returned to 19°C, male–male courtship behavior subsided in a few minutes. Besides synaptic vesicle recycling, *shi* is involved in endocytotic molecular trafficking that is essential for a variety of cellular processes (33–35). However, the quick onset of male–male courtship argues that disruption of synaptic transmission in the GAL4-positive neurons is likely to be the direct cause for this behavioral alteration.

Lee and Hall (24) recently demonstrated that *fru* mutations cause a previously unappreciated behavioral anomaly: high levels of head-to-head interactions between mutant males, which are considered as instances of aggression-like behavior that is distinct from, yet related to, male–male courtship. Interestingly, high levels of head-to-head interaction were observed also between C309/UAS-*shi*^{ts1} males after a temperature shift to 30°C. In typical head-to-head interactions, two males approached each other closely in a head-to-head position and each tapped at the other male's head with his forelegs (Fig. 2*F*, arrow). In a head-to-head position, they occasionally extended their wings and vibrated them unilaterally (Fig. 2*G*, arrow) or flicked both wings together (Fig. 2*H*, asterisk). The interacting pairs showed sporadic circling movements in which the pairs went around while keeping their heads in a close position. Flies stayed in head-to-head interactions for variable lengths of time, but the interaction most often lasted several seconds under the conditions used in this observation (20 males in a 35 × 10-mm Petri dish). Head-to-head interactions broke off when one of the flies turned around and ran away from the other or one made quick movements to orient himself to the other male's side or back. In either case, the head-to-head interactions generally were followed by male–male courtship.

To analyze these aberrant interactions among C309/UAS-*shi*^{ts1} males in a quantitative manner, five males with a distinct genotype were observed in Petri dishes at either 19 or 30°C, and the levels of courtship-chain formation and head-to-head interaction were determined (see *Materials and Methods*). Under the conditions used, C309/UAS-*shi*^{ts1} males exhibited a courtship chain during 37 ± 5% of a 10-min observation period, and at least one pair of flies exhibited a head-to-head interaction during 35 ± 9% of the observation period (Fig. 3). These male–male interactions were temperature- and *shi*^{ts1} expression-dependent. In control populations (C309/UAS-*shi*^{ts1} at 19°C and C309/+ or UAS-*shi*^{ts1}/+ at 30°C) flies displayed few intermale interactions: The head-to-head interactions occurred at most 0.2% of the time and lasted less than 1 sec, and courtship chains were never observed (Fig. 3).

The Temperature-Induced Male–Male Courtship Does Not Depend on Increased Sexual Attractiveness of C309/UAS-*shi*^{ts1} Males but on Their Increased Sexual Activities Toward Other Males. The temperature-induced male–male courtship could be due to an increase of sexual attractiveness of C309/UAS-*shi*^{ts1} males or, alternatively, their increased sexual activities toward other males. To distinguish between these two possibilities, pairs of a C309/UAS-*shi*^{ts1} male and a nontransformant (*w*) male were placed in courtship chambers and examined. As shown in Fig. 4 (Male–Male), C309/UAS-*shi*^{ts1} males showed a considerably higher courtship index toward *w* males in isolated pairs at 30 than at 19°C (43 ± 5 vs. 7 ± 3, $P = 1.1 \times 10^{-7}$). In contrast, *w* males showed little courtship behavior toward C309/UAS-*shi*^{ts1} males, and few head-to-head interactions were observed between *w* and C309/UAS-*shi*^{ts1} males regardless of temperature. A similar result was obtained when wild-type (Canton-S) males were used as a partner of C309/UAS-*shi*^{ts1} males (data not shown). Control males (C309/+ and UAS-*shi*^{ts1}/+) showed only low levels of courtship activity toward *w* males at 30°C (3 ± 1 and 4 ± 1; Fig. 4, Male–Male). The statistical differences between the courtship indices of C309/UAS-*shi*^{ts1} males and control males at 30°C were evident ($P = 4.3 \times 10^{-9}$ for C309/UAS-*shi*^{ts1} vs. C309/+ and $P = 8.6 \times 10^{-9}$ for C309/UAS-*shi*^{ts1} vs. UAS-*shi*^{ts1}/+). These results demonstrate that male–male courtship results from a temperature-dependent increase in sexual activity of C309/UAS-*shi*^{ts1} males toward other males but not from an increase in their sexual attractiveness.

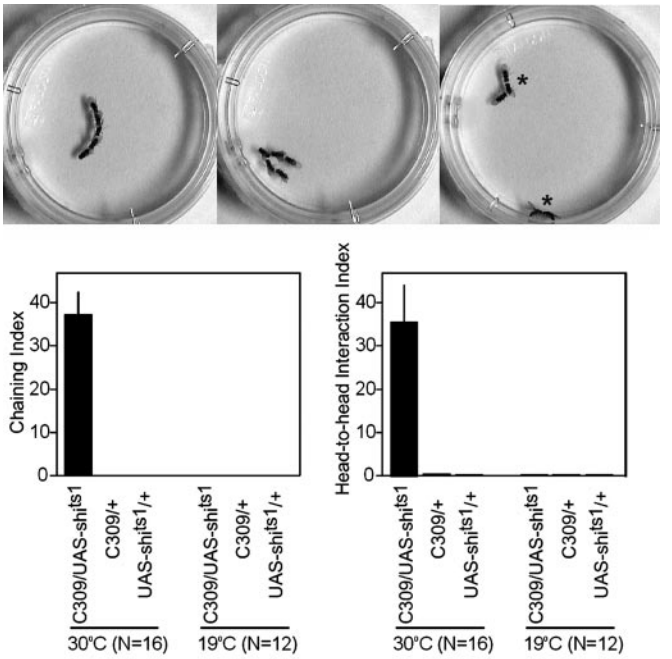

Fig. 3. Quantitative analysis of courtship chaining behavior and head-to-head interactions. Typical intermale interactions among five flies of C309/UAS-*shi*^{ts1} at 30°C. (*Upper Left*) A courtship chain composed of five males. (*Upper Center*) Four males showing courtship behavior and a male fly flicking both wings. (*Upper Right*) Two pairs of males exhibiting head-to-head interactions (asterisks). The average (±SEM) of chaining index (*Lower Left*) and head-to-head interaction index (*Lower Right*), which are determined for indicated genotypes of male flies at either 30 or 19°C (see *Materials and Methods*).

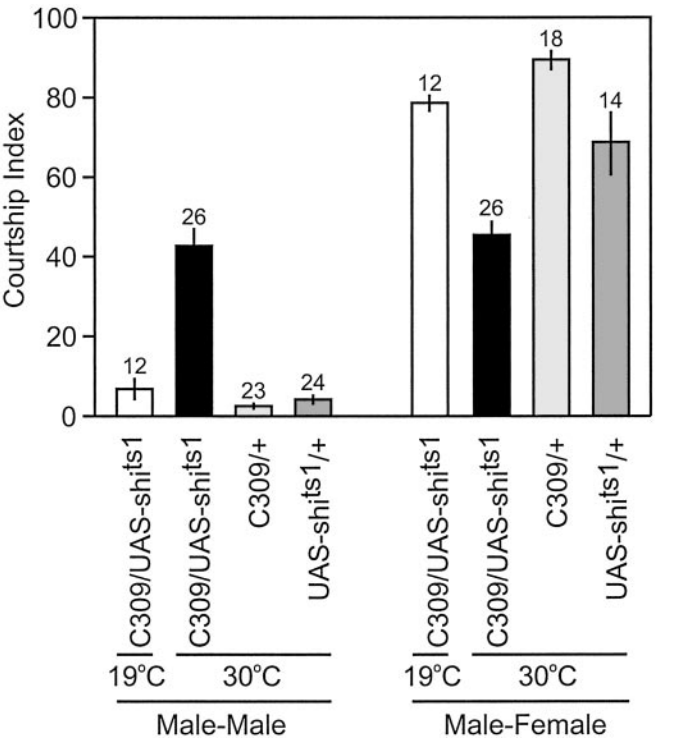

Fig. 4. Courtship index for isolated male–male (*Left*) or male–female (*Right*) pairs. The average (±SEM) of indicated number of measurements with different pairs is shown.

To see whether the evoked male–male courtship was caused by a general increase of sexual activity regardless of the partner's sex, C309/UAS-*shi*^{ts1} males were paired with virgin females, and their courtship index was scored. C309/UAS-*shi*^{ts1} males courted virgin females markedly less at 30 than at 19°C (45 ± 4 vs. 79 ± 2, $P = 4.4 \times 10^{-8}$), whereas control males (C309/+ and UAS-*shi*^{ts1}/+) retained relatively high levels of courtship activity at 30°C (89 ± 3 and 69 ± 8; Fig. 4. Male–Female). These data indicate that C309/UAS-*shi*^{ts1} males' high courtship index toward other males is not due to a general increase in their sexual activities but rather to a temperature-induced change in their mate preference.

In addition to their decreased courtship activity toward virgin females, C309/UAS-*shi*^{ts1} males had difficulty in copulating with wild-type (Canton-S) receptive females at 30°C. Only 6.5% (n = 31) of the C309/UAS-*shi*^{ts1} males paired with wild-type virgin females copulated during the 1-h observation period at 30°C (Fig. 5). All the C309/UAS-*shi*^{ts1} males at 19°C and most of the C309/+ males at 30°C mated during the observation period (100%, n = 14, and 90.5%, n = 21, respectively), indicating that the low mating success rate of the C309/UAS-*shi*^{ts1} males at 30°C depends on both temperature and targeted *shi*^{ts1} expression. In contrast, C309-directed expression of *shi*^{ts1} in the nervous system did not affect the receptiveness of virgin females. The mating success rate of C309/UAS-*shi*^{ts1} females with wild-type males at 30°C (88%, n = 25) was not significantly different from that of C309/UAS-*shi*^{ts1} females at 19°C (100%, n = 20; $\chi^2 = 2.57$, $P > 0.1$) or that of C309/+ at 30°C (96%, n = 25; $\chi^2 = 1.09$, $P > 0.2$) (Fig. 5).

Cholinergic Neurons Outside the MB Play a Critical Role in the Induction of Male–Male Courtship Behavior in C309/UAS-*shi*^{ts1}. Line C309 strongly expresses GAL4 in the MB intrinsic neurons (refs. 20, 21, and 26; Fig. 1). Because feminization of the MB neurons by ectopic expression of *tra*^{F} results in bisexual males (17, 18), it is possible that the temperature-induced male–male courtship observed in C309/UAS-*shi*^{ts1} is caused by conditional inactivation of the MB neurons. To test this possibility, four other enhancer-trap lines expressing GAL4 in the MB intrinsic neurons (Fig. 6) were examined. Line OK107 (26) labels almost all MB neurons (36), and lines C747 and c492b show expression in

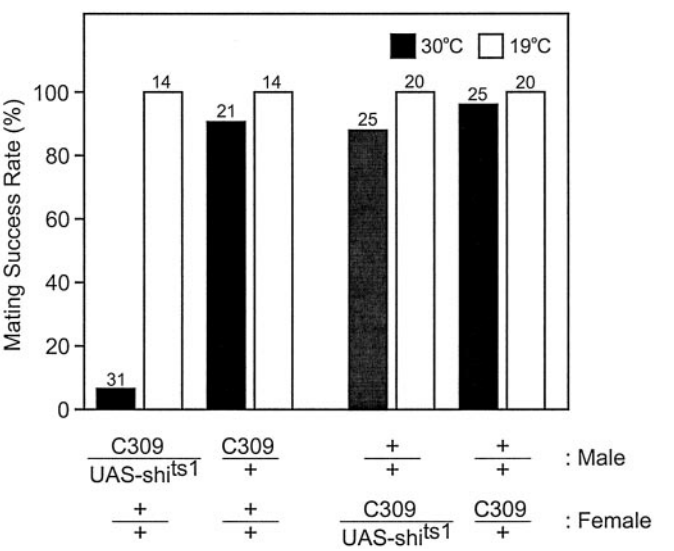

Fig. 5. Mating success rate of single male and female pairs. The percentage of pairs that have copulated in the 1-h observation period is shown. The number of independent pairs observed for each male–female combination is indicated.

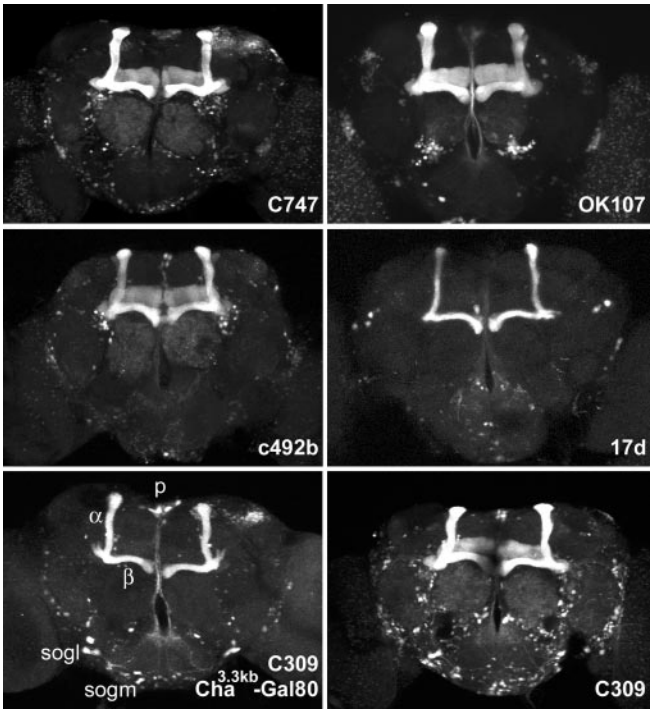

Fig. 6. GAL4 expression patterns in the adult brain of different enhancer trap GAL4 lines visualized by a GFP reporter gene. Lines C747, OK107, c492b, and 17d show prominent GFP expression in the MB intrinsic neurons. The introduction of a *Cha*$^{3.3kb}$-GAL80 construct into the C309/UAS-GFP flies suppresses GFP expression in the most part of the brain, whereas the pars intecerebralis (p), the MB α/β lobes, and neurons localized in the lateral and medial regions of the suboesophageal ganglion (sogl and sogm, respectively) remain GFP-positive.

large subsets of MB neurons (37, 38). The expression in line 17d is restricted to the MB neurons contributing to α/β lobes (39). These enhancer-trap GAL4 lines were crossed to UAS-*shi*^{ts1}, and the behavior of the progeny was examined. Expression of *shi*^{ts1} in MB neurons using these lines induced neither courtship-chain formation nor head-to-head interactions at 30°C (Table 1). The result argues against the possibility that conditional inactivation of the MB intrinsic neurons induces the male–male courtship behavior. Instead, it indicates that the behavioral modifications in C309/UAS-*shi*^{ts1} are caused by the temperature-induced disruption of synaptic transmission from the GAL4-positive neurons outside the MB.

To refine the search for these neurons, GAL80, another yeast transcription regulator, was used. The GAL80 protein antagonizes GAL4 activity by binding to the C-terminal activation domain of GAL4, thereby preventing interaction between GAL4 and the transcriptional machinery (40). Lee and Luo (36) reported that GAL80 efficiently antagonizes GAL4 activity in *Drosophila* without showing adverse effects on development or behavior. As a first application of GAL80 to the system, the involvement of cholinergic neurons in the induction of male–male courtship was examined. For this purpose the 3.3 kb of 5′-flanking DNA of the choline acetyltransferase gene (*Cha*), which directs gene expression in large subsets of cholinergic neurons (23, 41), was fused to the GAL80 gene, and the resultant construct (*Cha*$^{3.3kb}$-GAL80) was introduced into line C309. As judged by the GFP reporter gene expression, targeted expression of GAL80 suppressed the GAL4 activity in particular CNS neurons that presumably are cholinergic (Fig. 6 *Bottom Left* and *Bottom Right*). The number of GFP-positive neurons in the MB, AL, central complex, and optic lobes of the C309/UAS-GFP flies was reduced significantly, whereas the pars intecerebralis and MB intrinsic neurons contributing to the MB α/β lobes were still GFP-positive. GFP expression in the neurons with large cell bodies that were localized in the lateral and medial parts of the suboesophageal ganglion was not affected in the presence of the *Cha*$^{3.3kb}$-GAL80 construct (Fig. 6 *Bottom Left* and *Bottom Right*). GFP expression observed in the gustatory neurons of the labial palps and leg tarsal segments (Fig. 1 *C* and *D*) was suppressed by targeted GAL80 expression (data not shown), as expected from the previous observation that the 3.3-kb *Cha* regulatory DNA directs gene expression in most if not all chemosensory neurons in the peripheral nervous system (23, 41). Concomitant with the further restriction of the GAL4 activity in C309 by the *Cha*$^{3.3kb}$-GAL80 construct, the temperature-induced courtship-chain formation and head-to-head interactions were suppressed completely (Table 1). This result strongly suggests that, among the GAL4 positive neurons in C309, conditional disruption of the cholinergic neurons where the GAL4 activity was suppressed by the *Cha*$^{3.3kb}$-GAL80 construct plays a critical role in the induction of aberrant male–male courtship behavior.

Table 1. Male–male interactions in different GAL4/UAS-*shi*^{ts1} males at 30°C

GAL4 line	*N*	Chaining index	Head-to-head interaction index
C309	16	37.2 ± 5.5	35.5 ± 8.7
C747	10	0	1.4 ± 0.3
OK107	10	0	0.4 ± 0.04
c492b	10	0	0.3 ± 0.2
17d	10	0	0.4 ± 0.2
C309; *Cha*$^{3.3kb}$-GAL80	10	0	0.4 ± 0.06

Discussion

Most genetic variants in *Drosophila* showing aberrant male–male courtship have nervous systems that are modified irreversibly due to genetically disturbed developmental programs (6, 7, 9, 11, 12, 17, 18). The male–male courtship reported here is distinctive in that it can be turned on or off at different temperatures and is therefore not a consequence of abnormal development. Inducible male–male courtship behavior has been observed when flies containing a mini-*w* gene under the control of *hsp70* heat-shock promoter are heat-shocked at 37°C for 1 h or more (13, 14). However, the behavior reported here cannot be an artifact of any w^+ markers used in these experiments, because the temperature of induction is too subtle (30°C), and the behavior appears too quickly (a few minutes). Furthermore, the behavior entirely depends on the GAL4 and GAL80 constructs that are used to express or suppress anatomically specific *shi*^{ts1} expression (Figs. 3 and 4 and Table 1). Another interesting example of conditional male–male courtship behavior is seen with male flies carrying a reciprocal translocation between X (2E) and third chromosomes (97A) (42). The sexual activity of these mutant males lasts only as long as they are exposed to light, and the aroused males display courtship toward other males as well as females. The genes and neural mechanisms underlying this light-dependent change in male courtship behavior remain unknown.

Because disruption of synaptic transmission evokes male–male courtship, it is plausible that the relevant transmission is involved in inhibitory systems that ordinary suppress courtship behavior toward other males. In *Drosophila*, most of the chemical substances, including antiaphrodisiac pheromones that act during courtship, are detected mainly by direct contact during tapping and licking the partners with forelegs and the proboscis, respectively (43). C309 directs gene expression in small groups of gustatory neurons in the labial palps of the proboscis and the

tarsal segments of the leg (Fig. 1 *C* and *D*). When the GAL4 activity of cholinergic neurons, including the gustatory neurons of interest, was suppressed in line C309 by targeted GAL80 expression, the male–male courtship was concomitantly suppressed. Thus, one plausible explanation is that conditional disruption of synaptic transmission from GAL4-positive gustatory neurons in C309 deprives males of their ability to detect antiaphrodisiac pheromones produced by males. It is worth determining whether C309/UAS-*shi*^{ts1} males have defects in sensing the known antiaphrodisiac hydrocarbon molecules such as 7-tricosene (44, 45) in a temperature-dependent manner.

C309/UAS-*shi*^{ts1} males displayed less sexual activity toward receptive virgin females at 30 than at 19°C (Fig. 4). Therefore the loss of ability to detect antiaphrodisiac pheromones at the restrictive temperature can be only a partial explanation of their behavior. An alternative possibility is that disruption of a set of CNS neurons involved in the interpretation of gustatory and/or olfactory sensory information leads to changes in sexual activity toward both sexes. Interestingly, the courtship phenotype of C309/UAS-*shi*^{ts1} male at the restrictive temperature is very similar to that of *fru* mutants; they show homosexual courtship, court females at subnormal levels (5, 6), have defects in attempted copulation (3), and show head-to-head interactions (19). *fru* is required for the development of CNS neurons that are responsible for male sexual behavior (46, 47). It is possible that CNS neurons related to the *fru*-dependent neuronal circuits are conditionally perturbed in C309/UAS-*shi*^{ts1} males at the restrictive temperature.

The GAL4 expression pattern of line C309 is rather complex, and it is not possible at present to pinpoint the neurons responsible for the induction of male–male courtship. To address this issue, two approaches were taken. First, GAL4 lines showing overlapping expression patterns with line C309 were examined to determine whether they exhibited a behavioral phenotype similar to that shown by C309. Second, the GAL4 activity in C309 was restricted further to smaller subsets of neurons by targeted GAL80 expression, and its effect on the male–male courtship behavior was observed. The results of these experiments suggest that, among GAL4-positive neurons in the C309 line, conditional perturbation of cholinergic neurons outside the MB plays a critical role in the induction of male–male courtship. Further refinement of the critical neurons by analyzing different GAL4 lines with appropriate GAL80 constructs will shed light on the mechanisms controlling sexual orientation and other aspects of reproductive behavior in *Drosophila*.

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